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**13. ABSTRACT** *(Maximum 200 Words)*

Prostate cancer is now the most common cancer and the second most common cause of death from cancer, among men in the United States. Angiogenesis is required for prostate tumor survival, growth, and metastasis. We proposed to design and synthesize novel Hunter-Killer Peptides (HKPs), each representing a hybrid of an angiogenesis-targeting peptide and a mitochondrial membrane-disrupting peptide. While made to be *non-toxic* in the circulation, the HKPs will be preferentially *toxic* to mitochondria once internalized into angiogenic cells, via the targeting domain. We report here and in Ellerby et al., *Nature Medicine*, 5, 1032-1038, 1999, that while our prototypes contain only 21 and 26 amino acid residues, they are selectively toxic to angiogenic endothelial cells and show strong anti-cancer activity in mice (breast carcinoma xenografts). Work is underway to evaluate the HKPs for efficacy and toxicity in the TRAMP (transgenic adenocarcinoma mouse prostate) model for prostate cancer. The central theme of this research is to develop and appraise this new chemotherapy with the goal of producing both a safer, and more effective, treatment of advanced prostate cancer.

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# Introduction

The subject of the research is prostate cancer. The purpose of the research is the development of a more effective and less toxic treatment for prostate cancer. A major limitation of the currently used chemo-therapeutic agents is that these are the drugs with the narrowest therapeutic index in all of medicine. Thus, an effective dose of a wide variety of anti-cancer agents is restricted by their non-selective, highly toxic effect on normal tissues. Specifically, we proposed to design short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. We report here and in Ellerby *et al.*, *Nature Medicine*, **5**, 1032-1038, 1999, that while our prototypes contain only 21 and 26 amino acid residues, they are selectively toxic to angiogenic endothelial cells and show strong anti-cancer activity in mice.

# Body

Our work to date in designing, synthesizing, and testing Hunter-Killer Peptides (HKPs) is described in the accompanying reprint by Ellerby *et al.*, *Nature Medicine*, **5**, 1032-1038, 1999, in the appendix.

**Statement of Work**

**Task 1. Design and synthesis of Hunter-Killer peptides (HKPs) (months 0-2, year1).**

-We will use CNGRC peptide of Arap *et al.*, 1998 for the hunter domain for our initial studies.
-We will use 4-6 of the peptide designs of Javadpour *et al.*, 1996 for the killer domain to begin our studies.
-Both the all D- and all L-amino acid form (for the killer domain) of the HKPs will be made.
-The resulting HKPs will be synthesized and cyclized to HPLC purity by Anaspec (San Jose, CA).
-Depending upon the results of Task 2 and 3, we will proceed to optimize successful candidates, or search for new candidates through the use of function-based peptide design principles.

*Goal- The overall goal of this task is to acquire HKPs for use in Task 2 and 3.*

**Task 2. Evaluate the efficacy and toxicity of the HKPs in vitro (months 2-4, year 1).**

-We will test the HKPs toxicity to several mammalian cell lines, including 3T3s, through cell death studies in tissue culture.
-We will test the HKPs ability to disrupt mitochondrial membranes through swelling assays.
-The maximum concentration tolerated before any signs of mammalian cell death will be used with the minimum concentration required to swell mitochondria to compute a therapeutic index.

*Goal- The overall goal of this task is to characterize the efficacy and toxicity of the HKPs and arrive at a therapeutic index which then will act as a guide as to the concentrations of the HKPs to use in vivo.*

**Task 3. Evaluate the efficacy and toxicity of the HKPs in vivo (months 4-12, year 1).**

-We will prepare tumors using human prostate cell lines.

-We will initiate tumor treatment studies with HKPs in nude mice and C57BL/6 mice. Treatments will be given locally, intraperitoneally or systemically. We will perform tumor volume, survival, and histopathological studies.

-Then we will test HKPs against primary and metastatic prostate cancer in the TRAMP transgenic mouse model of prostate cancer.

-We will perform tumor volume and histopathological studies to quantify the effect of HKPs on tumors and metastatic lesions. In addition, survival studies will be performed.

**Progress on Year 01 Goals:**

As noted above, all our work to date has been summarized in Ellerby *et al.*, *Nature Medicine*, **5**, 1032-1038, 1999, included in the appendix. The key research accomplishments are discussed below. We completed Task 1 above, having developed 3 HKPs. We have completed Task 2 above, in that we tested 3 HKPs for efficacy and toxicity in tissue culture models of angiogenesis, mitochondrial swelling studies, and in cell-free systems. We have completed Task 3 for xenografts of human 435 breast carcinomas and human melanoma, and are in the process of establishing human prostate tumors in nude mice.

In we have conducted a pilot study with an HKP composed of $_D$(KLAKLAK)$_2$ for the killer domain and a normal prostate gland targeting sequence (proprietary) for the hunting domain to determine if this HKP could be used to reduce the size of the normal prostate. This organ ablation/reduction technology would be very useful for the treatment of benign prostatic hyperplasia to reduce the size of a pre-cancerous enlarged prostate, without the need for surgery and its complications, such as urinary incontinence.

In our experiment, 10 BALB/c mice were given a one-time injection of HKP. The preliminary result shows that the prostate glands from mice in the HKP treated group (5 animals/group) underwent significant apoptosis, while the prostate glands from the control group (5 animals/group) remained unchanged (Fig. 1). There were no apparent side effects of this procedure such as death, lethargy, etc., and tissue from liver, kidney, etc., appeared free of toxic effect (data not shown). This pilot study offers a first glimpse at this potential therapy. The next step is to proceed to a full study.



Fig. 1. Histopathological section of mouse prostate gland demonstrating HKPs promise as a chemical prostatectomy. Normal prostate gland is shown in the left panel. Prostate gland treated with a single dose (200_ g) of an HKP having a hunter domain specific for normal prostate vasculature. Destruction of the gland is evident with widespread apoptosis as seen in the left panel.
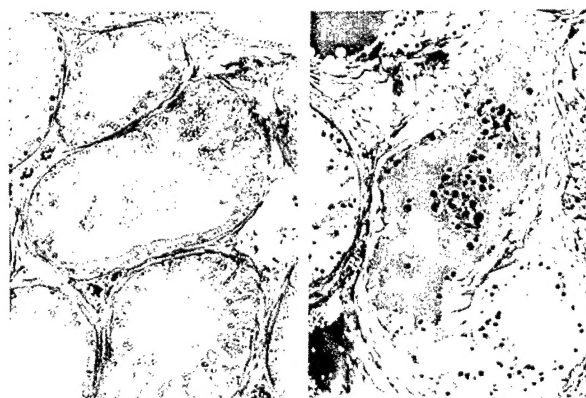
**Key Research Accomplishments**

(1) **We successfully designed and tested a Hunter-Killer Peptide, HKP-1, CNGRC-GG-$_D$(KLAKLAK)$_2$.** We evaluated HKP-1 for efficacy and toxicity in a tissue culture model of angiogenesis developed by our laboratory. The *in vitro* therapeutic index, the ratio of [control peptide (50:50 mixture of uncoupled hunter and killer)]/[HKP] required to kill angiogenic endothelial cells, for HKP-1 was found to be 100. We also evaluated HKP-1 in a nude mouse model of human breast carcinoma. Tumor volumes were reduced and survival was extended. This newly designed peptide will now be evaluated in a nude mouse model of prostate carcinoma. See accompanying reprint (Ellerby *et al.*, *Nature Medicine*, 5, 1032-1038, 1999, in the appendix.

(2) **We successfully designed and tested a second more effective/less toxic Hunter-Killer Peptide, HKP-2, ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.** We substituted the targeting domain ACDCRGDCFC (Hunter) for the targeting domain CNGRC used in our prototype HKP-1. We evaluated HKP-2 for efficacy and toxicity in a tissue culture model of angiogenesis developed by our laboratory. The *in vitro* therapeutic index, the ratio of [control peptide (50:50 mixture of uncoupled hunter and killer)]/[HKP] required to kill angiogenic endothelial cells, for HKP-2 was found to be 4-5 times greater than that for HKP-1. We also evaluated HKP-2 in a nude mouse model of human breast carcinoma. Tumor volumes were reduced and metastasis was inhibited. This newly designed peptide will now be evaluated in a nude mouse model of prostate carcinoma. See accompanying reprint (Ellerby *et al.*, *Nature Medicine*, 5, 1032-1038, 1999, in the appendix.

(3) **We successfully designed and tested a third more effective/less toxic Hunter-Killer Peptide, HKP-3.** The *in vitro* therapeutic index for HKP-3 was found to be 1000-2000 times greater than that found for either HKP-1 or HKP-2 (Ellerby *et al.*, unpublished results). This newly designed peptide will now be evaluated in nude mouse models of human breast carcinoma and prostate carcinoma.

(4) **We evaluated HKP-1, CNGRC-GG-$_D$(KLAKLAK)$_2$, *in vivo* using a nude mouse model of human melanoma.** We performed a tumor study on nude mice bearing human melanoma tumors treated with HKP-1. Tumor volumes were significantly decreased in the HKP-1 treated animals (data not shown).

(5) **We obtained new evidence in support of our hypothesized mechanism of HKP activity.** We demonstrated in our *in vitro* cell culture model of angiogenesis that HKPs are *internalized* into targeted endothelial cells, using 2 complementary experimental approaches (Ellerby *et al.*, 1999). First, we detected biotintylated homing peptides within targeted cells. Second, through the use of cell fractionation and mass spectrometry, we showed that HKPs are internalized, and concentrate in mitochondria (rather than say the nucleus) (Ellerby *et al.*, 1999). We also demonstrated *in vitro* that HKPs *disrupt the negatively charged membranes of mitochondria*, early in the apoptosis induction process, using 3 complementary experimental approaches. First, using mitochondria-specific fluorescent dye, we showed that mitochondria adopt an abnormal morphology (osmotic swelling) early in the cell death process, before any other obvious morphological change occurs that would be indicative of early apoptosis, such

as "rounding-up." Second, using electron microscopy, we confirmed that early on in the apoptosis of the targeted endothelial cells, mitochondria appear to be the only adversely affected membraneous/organellar structure. Third, through the use of cell fractionation and mass spectrometry, we further confirmed that mitochondria are the target of HKPs.

**(6) We successfully tested a computer program designed to select optimum anti-mitochondrial peptides.** As proof of principle that anti-bacterial/mitochondrial peptides (killer domains) could be designed/chosen *in silico*, we created a computer program designed to select optimum mitochondrial membrane disrupting peptides. Specifically, the program automatically searched protein data bases and selected peptides/protein fragments according to a pre-defined set of optimal search ranges for peptide properties, including hydrophobic moment, percent helicity, isoelectric point, and the angle subtended by the charged residues. Out of thousands of possibilities, the computer selected a very limited number of amphipathic sequences (~20), including some peptides known to be excellent anti-bacterial peptides (e.g magainin), and also some novel sequences, including the biologically relevant neuropeptide substance P. Mitochondrial swelling studies, and a mitochondria-dependent cell-free system of apoptosis, experimentally confirmed that substance P was indeed an excellent mitochondrial membrane disruption agent (data not shown). This behavior of substance P has never been described before, and may have application in the study of Alzheimer's disease, where the selective loss of substance P producing neurons has been well documented. Thus our pilot computer program has generated some novel, and potentially disease-relevant, killing domain candidates.

**Reportable Outcomes**

**Papers, manuscripts, abstracts, presentations**

(1) Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Del Rio G, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R (1999) Anti-cancer activity of targeted pro-apoptopic peptides. Nature Medicine 5(9):1032-1038.
(2) H. Michael Ellerby, Sannamu Lee, Taira Kiyota, Rebbecca L. Andrusiak, Lisa M. Ellerby, Stephanie Propp, Dale E. Bredesen, and Renata Pasqualini. "*De Novo* synthesized Small Globular Protein is Anti-Tumorigenic and a Model for Future Chemotherapy." Manuscript in preparation.

**Patents and licenses applied for and/or issued**
H. Michael Ellerby, Dale E. Bredesen, Renata Pasqualini, and Erkki Ruoslahti (1999). "Targeted Pro-Apoptotic Peptides for Cancer Therapy."

**Degrees obtained that are supported by this award**
None.

**Development of cell lines, tissue or serum repositories**
None.

**Informatics such as databases and animal models, etc**
None.

**Funding applied for based on work supported by this award**
None at this time.

**Employment or research opportunities applied for and/or received on experiences/training supported by this award**
None at this time.

## Conclusions

**Importance and/or Implications**

We have designed short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. We demonstrated in Ellerby *et al.*, *Nature Medicine*, 5, 1032-1038, 1999, that while our prototypes contain only 21 and 26 amino acid residues, they are selectively toxic to angiogenic endothelial cells and show strong anti-cancer activity in mice (xenografts of human breast carcinomas and melanomas). Work is underway to demonstrate the anti-cancer efficacy of HKPs in mouse models of human prostate cancer.

**Knowledge as a scientific or medical product**

The knowledge gained from these studies supported by the Department of Defense represents an important scientific and medical product in that HKPs are a new class of chemotherapuetic agents that can be modulated to obtain maximum therapeutic effect in the treatment of prostate cancer.

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## List of Personnel

**(9/1/98-8/31/99)**
Dale E. Bredesen
H. Michael Ellerby
Renata Pasqualini

## Appendices

Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Del Rio G, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R (1999) Anti-cancer activity of targeted pro-apoptopic peptides. Nature Medicine 5(9):1032-1038.

CV (2pg.), D. Bredesen.

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

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| California Institute of Technology, Pasadena, CA | B.S. | 1974 | Biology & Literature |
| Duke University Medical Center, Durham, NC | M.D. | 1977 | Medicine |

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## RESEARCH AND PROFESSIONAL EXPERIENCE

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| 6/78-7/80 | Duke University Medical Center | Resident, Medicine |
| 7/80-6/83 | University of California, San Francisco | Resident, Neurology |
| 7/83-6/87 | University of California, San Francisco | Clinical Instructor, Neurology |
| 1/85-1/86 | Laboratory of Dr. LY Jan (Drosophila Neurogenetics) Howard Hughes Medical Institute, San Francisco | Hughes Associate |
| 2/86-6/89 | UCSF, Laboratory of Dr. SB Prusiner (Neural Degenerative Disease) | NIH Postdoctoral Fellow |
| 7/87-2/89 | UCSF, Department of Neurology | Assistant Adjunct Professor |
| 3/89-6/94 | UCLA, Department of Neurology | Assistant Professor |
| 7/94-6/95 | UCLA, Department of Neurology | Associate Professor |
| 11/94-present | The Burnham Institute | Adjunct Professor |
| 3/96-present | UCSD, Neuroscience Department | Associate Adjunct Professor |
| 7/99-present | The Buck Center for Research in Aging | Professor; President and CEO |

## ACTIVITIES AND AWARDS

| | | | | |
|---|---|---|---|---|
| 1996 | Lou & Eleanor Gehrig Lectureship, Columbia Univ. | 1970-74 | Alfred P. Sloan Scholar, Caltech |
| 1993-95 | Elizabeth R. & Thomas E. Plott Chair, UCLA | 1974 | Graduation with Honor, Caltech |
| 1992 | Cotzias Award, American Parkinson Disease Assoc. | 1974 | McKinney Prize for Humanities, Caltech |
| 1984 | Outstanding Faculty Teacher Award | 1973 | NSF Summer Fellow, Caltech (Dr. R. Sperry, Psychobiology) |
| 1983 | Sandoz Award for Outstanding Neurology Resident | | |
| 1982-93 | Chief Resident, Neurology, UCSF | 1972 | Member, Board of Control, Caltech |
| 1977 | Trent Prize in the History of Medicine, Duke | 1972 | Research Asst., MIT (Dr. M. Wrighton, Inorg. Chemistry) |
| 1977 | Brody Scholar in the History of Neurosciences, Duke | 1971 | Research Asst., Caltech (Dr. H. Gray, Inorg. Chemistry) |
| 1975-77 | Mary Duke Biddle Scholar, Duke | | |

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# Anti-cancer activity of targeted pro-apoptotic peptides

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**We have designed short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. Although our prototypes contain only 21 and 26 residues, they were selectively toxic to angiogenic endothelial cells and showed anti-cancer activity in mice. This approach may yield new therapeutic agents.**

Tumor cell survival, growth and metastasis require persistent new blood vessel growth[1-3] (angiogenesis). Consequently, a strategy has emerged to treat cancer by inhibiting angiogenesis[4]. Peptides have been described that selectively target angiogenic endothelial cells[5-8]. Conjugates made from these peptides and the anti-cancer drug doxorubicin induce tumor regression in mice with a better efficacy and a lower toxicity than doxorubicin alone[8]. There is also a functional class of cell death-inducing receptors, or 'dependence receptors', which have embedded pro-apoptotic amino-acid sequences[9,10]. These peptide domains are required for apoptosis induction by these receptors. The peptide fragments are thought to be released into the cytosol as cleavage products of caspase proteolysis, where they induce or potentiate apoptosis through unknown mechanisms[9,10]. However, such peptides, and structurally similar pro-apoptotic antibiotic peptides, although they remain relatively non-toxic outside of eukaryotic cells, induce mitochondrial swelling and mitochondria dependent cell-free apoptosis[10,11].

There are more than 100 naturally occurring antibiotic peptides, and their *de novo* design has received much attention[12-14]. Many of these peptides are linear, cationic and $\alpha$-helix-forming. Some are also amphipathic, with hydrophobic residues distributed on one side of the helical axis and cationic residues on the other[15]. Because their cationic amino acids are attracted to the head groups of anionic phospholipids, these peptides preferentially disrupt negatively charged membranes. Once electrostatically bound, their amphipathic helices distort the lipid matrix (with or without pore formation), resulting in the loss of membrane barrier function[15,16]. Both prokaryotic cytoplasmic membranes and eukaryotic mitochondrial membranes (both the inner and the outer) maintain large transmembrane potentials, and have a high content of anionic phospholipids, reflecting the common ancestry of bacteria and mitochondria[15-19]. In contrast, eukaryotic plasma membranes (outer leaflet) generally have low membrane potentials, and are almost exclusively composed of zwitterionic phospholipids[16,18,20]. Many antibacterial peptides, therefore, preferentially disrupt prokaryotic membranes and eukaryotic mitochondrial membranes rather than eukaryotic plasma membranes.

If such nontoxic peptides were coupled to tumor targeting peptides that allow receptor-mediated internalization, the chimeric peptide would have the means to enter the cytosol of targeted cells, where it would be toxic by inducing mitochondrial-dependent apoptosis[10,11]. Thus, we designed targeted pro-apoptotic peptides composed of two functional domains. The targeting domain was designed to guide the 'homing' pro-apoptotic peptides to targeted cells and allow their internalization[8,21,22]. The pro-apoptotic domain was designed to be non-toxic outside of cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes.

### Design of the pro-apoptotic peptide

A computer-generated model and the sequence of one of our prototypes are shown in Fig. 1. For the targeting domain, we used either the cyclic (disulfide bond between cysteines) CNGRC peptide (Fig. 1) or the double-cyclic ACDCRGDCFC peptide (called RGD-4C), both of which have 'tumor-homing' properties[5,8] and for which there is evidence of internalization[8,21,22]. We synthesized this domain from all-L amino acids because of the presumed chiral nature of the receptor interaction. For the pro-apoptotic domain, we selected the synthetic 14-amino-acid peptide KLAKLAKKLAKLAK (Fig. 1), called $(KLAKLAK)_2$, because it killed bacteria at concentrations 1% of those required to kill eukaryotic cells[13]. We used the all-D enan-
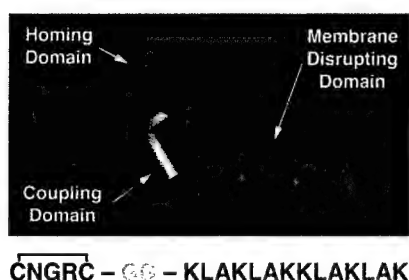
**Fig. 1** Computer-generated model and amino-acid sequence of CNGRC-GG$_D$(KLAKLAK)$_2$. This peptide is composed of a 'homing' domain (blue) and a membrane-disrupting (pro-apoptotic) domain (red hydrophilic and green hydrophobic residues), joined by a coupling domain (yellow).

tiomer $_D$(KLAKLAK)$_2$ to avoid degradation by proteases[12,23]. This strategy was possible because such peptides disrupt membranes by chiral-independent mechanisms[23,24]. We coupled the targeting (CNGRC or RGD-4C) and pro-apoptotic $_D$(KLAKLAK)$_2$ domains with a glycinylglycine bridge (Fig. 1) to impart peptide flexibility and minimize potential steric interactions that would prevent binding and/or membrane disruption.

### D(KLAKLAK)2 disrupts mitochondrial membranes

We evaluated the ability of $_D$(KLAKLAK)$_2$ to disrupt mitochondrial membranes preferentially rather than eukaryotic plasma membranes by mitochondrial swelling assays, in a mitochondria-dependent cell-free system of apoptosis, and by cytotoxicity assays[10]. There was morphological evidence of damage to mitochondrial membranes by electron microscopy. The peptide $_D$(KLAKLAK)$_2$ induced considerable mitochondrial swelling at a concentration of 10 µM (Fig. 2a). Mild swelling was evident even at 3 µM (data not shown), 1% the concentration required to kill eukaryotic cells (approximately 300 µM), as determined by the lethal concentration required to kill 50% of a cell monolayer (LC$_{50}$; Table 1). These results demonstrate that $_D$(KLAKLAK)$_2$ preferentially disrupts mitochondrial membranes rather than eukaryotic plasma membranes. Moreover, the peptide activated mitochondria-dependent cell-free apoptosis in a system composed of mitochondria suspended in cytosolic extract[10], as measured by characteristic caspase-3-processing from an inactive zymogen to active protease[25] (Fig. 2b). A non-α-helix-forming peptide, DLSLARLATARLAI (negative control), did not induce mitochondrial swelling (Fig. 2a), was inactive in the cell-free system (Fig. 2b) and was not lethal to eukaryotic cells[10]. We also analyzed morphologic alterations in isolated mitochondria

by electron microscopy. The peptide $_D$(KLAKLAK)$_2$ induced abnormal mitochondrial morphology, whereas the control peptide DLSLARLATARLAI did not (Fig. 2c).

### Targeted pro-apoptotic peptides induce apoptosis

We evaluated the efficacy and specificity of CNGRC-GG-$_D$(KLAKLAK)$_2$ in KS1767 cells, derived from Kaposi sarcoma[26,27] (Fig. 3a–d), and MDA-MB-435 human breast carcinoma cells[5,8] (Table 1). We used KS1767 cells because they bind the CNGRC targeting peptide just as endothelial cells do. This may relate to the endothelial origin of the KS1767 cells[27]. We used MDA-MB-435 cells as negative control cells because they do not bind the CNGRC targeting peptide[8]. Although CNGRC-GG-$_D$(KLAKLAK)$_2$ was considerably toxic to KS1617 cells, an equimolar mixture of uncoupled CNGRC and $_D$(KLAKLAK)$_2$ (negative control), or $_D$(KLAKLAK)$_2$ alone, was much less toxic, indicative of a targeting effect (Table 1). In contrast, CNGRC-GG-$_D$(KLAKLAK)$_2$ was not very toxic to MDA-MB-435 cells, which do not bind the CNGRC peptide (Table 1). The other targeted peptide (RGD-4C)-GG-$_D$(KLAKLAK)$_2$, showed toxic effects similar to those of CNGRC-GG-$_D$(KLAKLAK)$_2$ on KS1617 cells, whereas an equimolar mixture of uncoupled RGD-4C and $_D$(KLAKLAK)$_2$, used as a negative control, was not very toxic (Table 1; Fig. 3c–d).

Although evidence for internalization of CNGRC and RGD-4C into the cytosol of cells has been published[5,8,21,22], we directly demonstrated internalization using biotin-labeled peptides. CNGRC–biotin, but not untargeted CARAC–biotin, was internalized into the cytosol of cells (Fig. 3e–f). We also obtained direct evidence for internalization from experiments based on cell fractionation and mass spectrometry. CNGRC-GG-$_D$(KLAKLAK)$_2$, but not CARAC-GG-$_D$(KLAKLAK)$_2$, was indeed internalized and could be detected in mitochondrial as well as cytosolic fractions (data not shown).

Next, we evaluated the efficacy and specificity of CNGRC-GG-$_D$(KLAKLAK)$_2$ in a tissue culture model of angiogenesis[28]. During angiogenesis, capillary endothelial cells proliferate and migrate[1,2]. Cord formation is a type of migration that can be studied *in vitro* by a change in endothelial cell morphology from the usual 'cobblestones' to chains or cords of cells[27]. We tested the effect of CNGRC-GG-$_D$(KLAKLAK)$_2$ on normal human dermal microvessel endothelial cells (DMECs) in the angiogenic conditions of proliferation and cord formation and in the angiostatic condition of a monolayer maintained at 100% confluency.

The treatment of DMECs with 60 µM CNGRC-GG-$_D$(KLAKLAK)$_2$ led to a decrease in the percent viability over time compared with that of untreated controls, in the conditions of proliferation (Fig. 4a) or cord formation (Fig. 4b). In contrast, treatment with the untargeted peptide $_D$(KLAKLAK)$_2$ as a negative control led to a negligible loss in viability. Furthermore, the LC$_{50}$ for proliferating or migrating DMECs treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ was 10% of the LC$_{50}$ for angiostatic DMECs maintained in a monolayer at 100% confluency (Table 1). This result indicates that CNGRC-GG-$_D$(KLAKLAK)$_2$ kills cells in angiogenic but not angiostatic conditions. The LC$_{50}$ for the untargeted control $_D$(KLAKLAK)$_2$ in angiogenic con-

**Table 1** LC$_{50}$ (µM) for eukaryotic cells treated with targeted pro-apoptotic peptides

| | DMEC | | | KS1767 | MDA-MB-435 |
|---|---|---|---|---|---|
| | Angiostatic | Angiogenic | | | |
| | | Proliferation | Cord Form | Proliferation | Proliferation |
| $_d$(KLAKLAK)$_2$ | 492 | 346 | 368 | 387 | 333 |
| CNGRC-GG-$_D$(KLAKLAK)$_2$ | 481 | 51[a] | 34[a] | 42[a] | 415 |
| (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ | – | – | – | 10[a] | – |

Results are means of three independent experiments. [a]$P<0.03$, t-test.
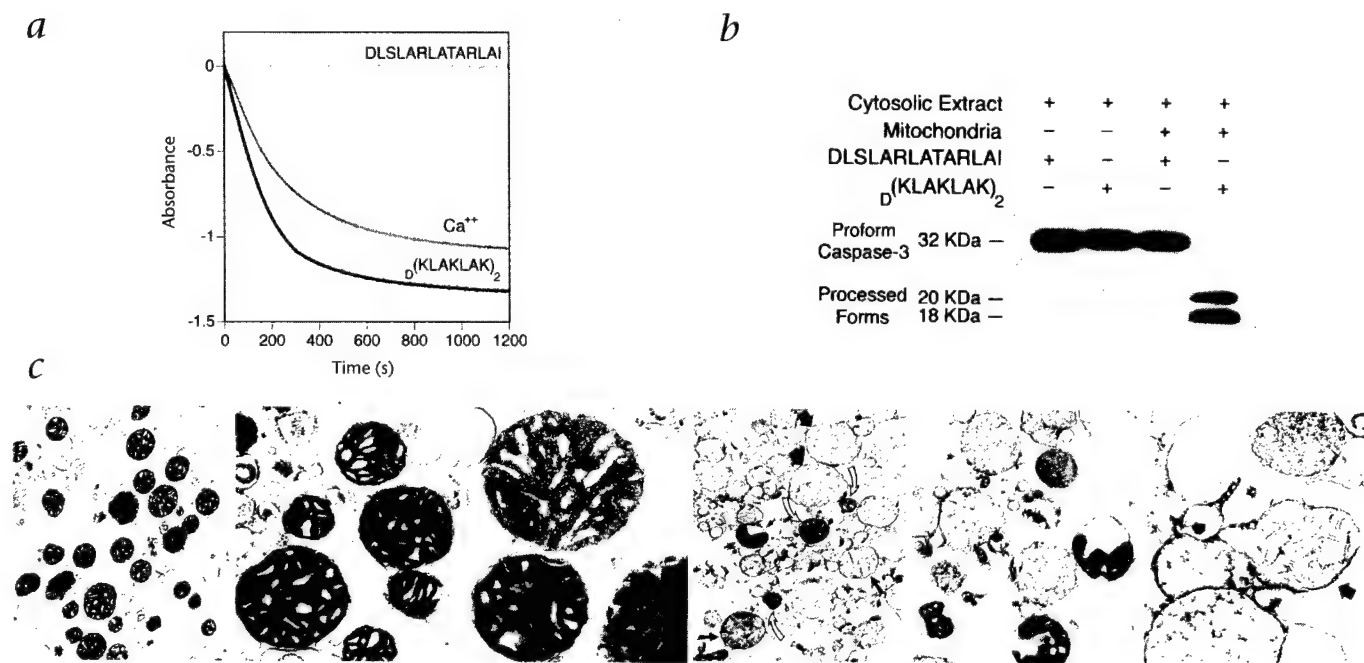
**Fig. 2** $_D$(KLAKLAK)$_2$ disrupts mitochondrial membranes. **a,** $_D$(KLAKLAK)$_2$ or Ca$^{2+}$ (positive control) induced mitochondrial swelling, whereas the non-α-helix-former DLSLARLATARLAI (negative control) did not, as shown by mitochondrial swelling curves (optical absorbance spectrum). **b,** $_D$(KLAKLAK)$_2$ activates cell-free apoptosis in a system composed of normal mitochondria and cytosolic extract, but DLSLARLATARLAI does not. An immunoblot of caspase-3 cleavage from proform (32-kDa) to processed forms (18- and 20-kDa) demonstrates a mitochondria-dependent cell-free apoptosis (left margin, sizes). Results were reproduced in two independent experiments. **c,** Morphologic alterations in isolated mito-chondria analyzed by electron microscopy. Mitochondria incubated for 15 min with 3 μM DLSLARLATARLAI show normal morphology (left panels). In contrast, mitochondria incubated for 15 min with 3 μM $_D$(KLAKLAK)$_2$ show extensive morphological changes. The damage to mitochondria progressed from the stage of focal matrix resolution (short black arrow), through homogenization and dilution of condensed matrix content with sporadic remnants of cristae (long black arrows), to extremely swollen vesicle-like structures (thick black arrows; bottom right, higher magnification); few mitochondria had normal morphology (open arrows). Ultrathin sections are shown. Original magnification, ×4,000–×40,000.

ditions was similar to the LC$_{50}$ for CNGRC-GG-$_D$(KLAKLAK)$_2$ under angiostatic conditions. An equimolar mixture of uncoupled $_D$(KLAKLAK)$_2$ and CNGRC, a non-targeted form CARAC-GG-$_D$(KLAKLAK)$_2$, and a 'scrambled' form, CGRNC-GG-$_D$(KLAK-LAK)$_2$, all gave results similar to those of $_D$(KLAKLAK)$_2$.

We also studied the mitochondrial morphology of DMECs in the condition of proliferation, after treatment with 60 μM CGRNC-GG-$_D$(KLAKLAK)$_2$ or untargeted $_D$(KLAKLAK)$_2$. The mitochondria in intact DMECs treated for 24 hours with the equimolar mixture CNGRC and $_D$(KLAKLAK)$_2$ remained morphologically normal (Fig. 4d), whereas those treated with CGRNC-GG-$_D$(KLAKLAK)$_2$ showed altered mitochondrial morphology, evident in approximately 80% of cells (Fig. 4e), before the cells rounded-up. Ultimately, the DMECs treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ showed the classic morphological indicators of apoptosis, including nuclear condensation and fragmentation, as seen at 72 hours (Fig. 4 f and g)(ref. 10). Apoptotic cell death (Fig. 4g) was confirmed with an assay for

**Fig. 3** CNGRC-GG-$_D$(KLAKLAK)$_2$ and (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ induce apoptosis. **a,** KS1767 cells treated with 100 μM of non-targeted CARAC-GG-$_D$(KLAK-LAK)$_2$ (negative control) remain unaffected after 48 h. **b,** KS1767 cells treated with 100 μM of CNGRC-GG-$_D$(KLAKLAK)$_2$ undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident. **c,** KS1767 cells treated with 10 μM of an equimolar mixture of (RGD-4C) and $_D$(KLAKLAK)$_2$ (negative control) remain unaffected after 48 h. **d,** KS1767 cells treated with 10 μM of (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident. Scale bar represents 250 μm. **e** and **f,** KS1767 cells treated with 100 μM of CNGRC-biotin (e) or CARAC-biotin (f) for 24 h and subsequently
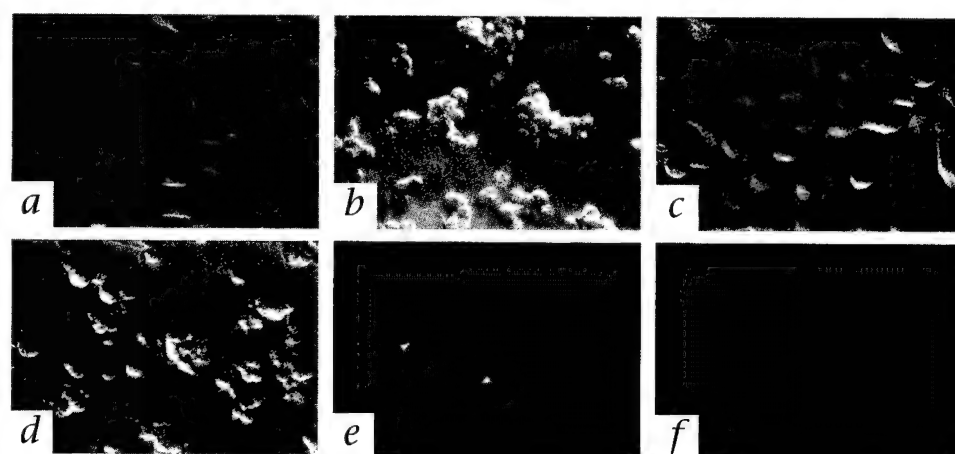


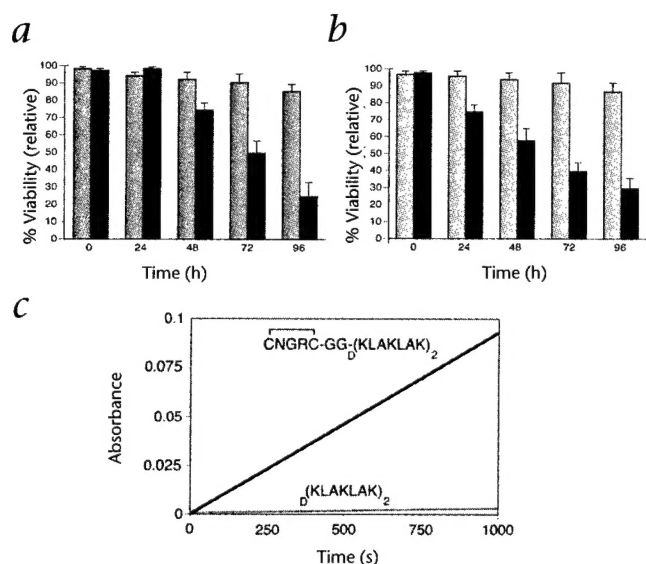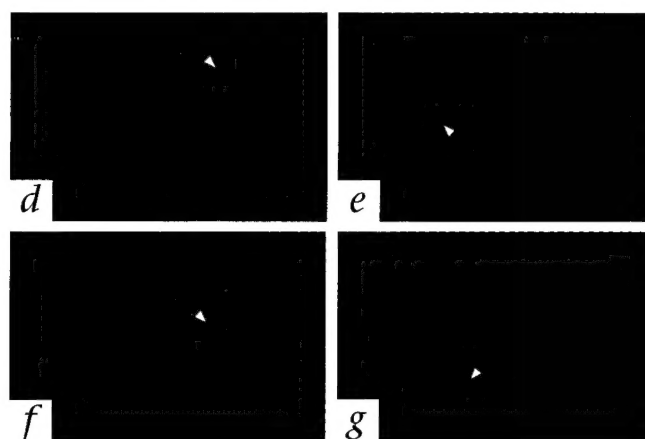treated with streptavidin FITC demonstrate internalization of CNGRC-biotin, but not CARAC-biotin, into the cytosol.

*a*

*b*

*c*

*d*

*e*

*f*

*g*

**Fig. 4** CNGRC-GG-$_D$(KLAKLAK)$_2$ induces apoptosis and mitochondrial swelling in DMECs. **a**, Proliferating DMECs treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ (filled bars) lose viability (apoptosis) over time ($P < 0.02$), but those treated with the control peptide $_D$(KLAKLAK)$_2$ (gray bars) do not ($P < 0.05$). **b**, Cord-forming DMECs lose viability (apoptosis) over time (filled bars), but those treated with $_D$(KLAKLAK)$_2$ (gray bars) do not ($P < 0.05$). **c**, Apoptotic cell death was confirmed with an assay for caspase 3 activity, as shown by the hydrolysis of DEVD-pNA with time. Results were reproduced in three independent experiments. **d**, Proliferating DMECs show normal nu-clear (blue) and mitochondrial (red) morphology after 24 h of treatment with a mixture of 100 μM $_D$(KLAKLAK)$_2$ and CNGRC. **e–g**, Proliferating DMECs treated with 100 μM CNGRC-GG-$_D$(KLAKLAK)$_2$. After 24 h (*e*), cells show normal nuclear (blue) but abnormal mitochondrial (red) morphology. Mitochondrial swelling and dysfunction is shown by a decrease in fluores-cence intensity and a change in morphology from an extended lace-like net-work to a condensed clumping of spherical structures. Classic morphological indicators of mid- to late apoptosis (for example, condensed and fragmented nuclei) are evident at 48 h (*f*) and 72 h (*g*) (arrow).

caspase 3 activity[10]. We also tested a caspase inhibitor for its ef-fect on cell death induced by CNGRC-GG-$_D$(KLAKLAK)$_2$. We used Kaposi sarcoma cells, as these cells bind CNGRC. The in-hibitor zVAD.fmk, at a concentration (25 μM) that inhibits cas-pases but not non-caspase proteases, inhibited the cell death induced by CNGRC-GG-$_D$(KLAKLAK)$_2$ (data not shown). This result is compatible with the earlier demonstration that the

CNGRC-GG-$_D$(KLAKLAK)$_2$ peptide is pro-apoptotic. Although the relatively early mitochondrial swelling is consistent with the putative mechanism of action, that is, a direct activation of the apoptotic machinery, we cannot rule out the possibility that the peptides actually kill by inducing some irreversible damage to cells which then activates the apoptotic program.

In addition to the fluorescence studies shown above, we stud-ied cultured cells by electron microscopy to confirm that CNGRC-GG-$_D$(KLAKLAK)$_2$ induces abnormal mitochondrial morphology in intact cells (Fig. 5). Kaposi sarcoma-derived KS1767 cells treated with the control peptide CARAC-GG-$_D$(KLAKLAK)$_2$ for 72 hours showed no overall changes, with no or very minor changes in the mitochondria (Figs. 5a–c). In con-trast, the mitochondria in KS1767 cells incubated for 12 hours
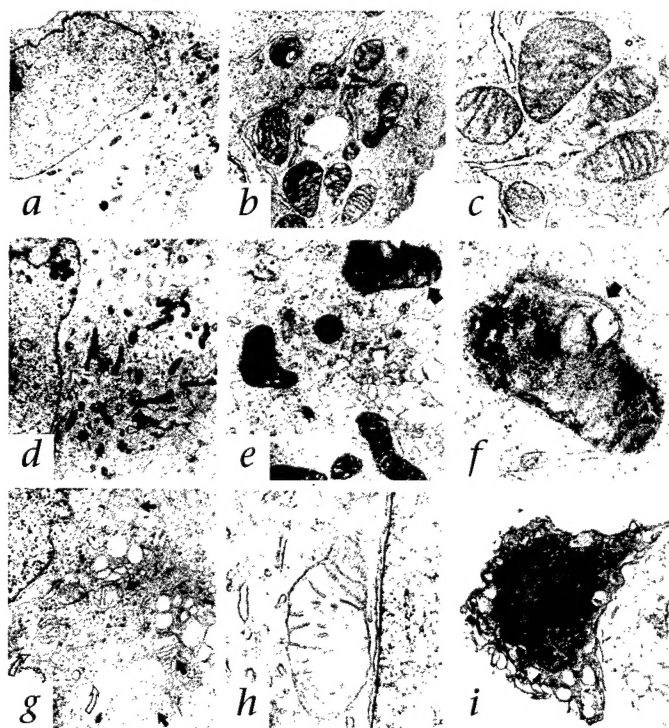


*a*

*b*

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*h*

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**Fig. 5** Electron microscopic studies of cultured cells. *a–c*, KS1767 cells treated with 100 μM CARAC-GG-$_D$(KLAKLAK)$_2$ for 72 h show the repre-sentative ultrastructural details of normal cells, with no or negligible changes seen in the mitochondria. Original magnifications: **a**, ×4,000; **b**, ×25,000; **c**, ×45,000. **d–f**, In contrast, the mitochondria in KS1767 cells incubated for 12 h with 100 μM CNGRC-GG-$_D$(KLAKLAK)$_2$ begin to show a condensed appearance and vacuolization despite a relatively normal cell morphology (black arrows). Original magnifications: *d*, ×12,000; *e*, ×20,000; *f*, ×45,000). *g* and *h*, Progressive damage to KS1767 cells is evident after 24 h, when many mitochondria show typi-cal large matrix compartments and prominent cristae, ultrastructural features of low level of oxidative phosphorylation. Original magnifica-tions: **g**, ×12,000; **h**, ×40,000. Some of the swollen mitochondria (*g*, black arrows) are similar in appearance to those in isolated mito-chondria treated with 100 μM $_D$(KLAKLAK)$_2$ (Fig. 2c, bottom right). *i*, In some cells, this process progressed to a final stage, with extensive vac-uolization and the pyknotic, condensed nuclei typical of apoptosis. Original magnification, ×8,000.

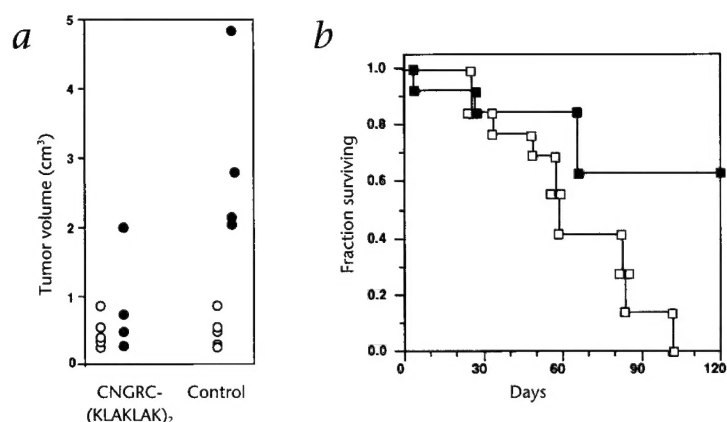**Fig. 6** Treatment of nude mice bearing MDA-MB-435-derived human breast carcinoma xenografts with CNGRC-GG-$_D$(KLAKLAK)$_2$. **a**, Tumors treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ are smaller than control tumors treated with CARAC-GG-$_D$(KLAKLAK)$_2$, as shown by differences in tumor volumes between day 1 (O) and day 50 (●). $P$ = 0.027, $t$-test. One mouse in the control group died before the end of the experiment. **b**, Mice treated with CNGRC-GG- $_D$(KLAKLAK)$_2$ (■) survived longer than control mice treated with an equimolar mixture of $_D$(KLAKLAK)$_2$ and CNGRC (□), as shown by a Kaplan-Meier survival plot ($n$ = 13 animals/group). $P$ < 0.05, log-rank test.

Treatment of nude mice bearing human tumor xenografts with CNGRC-GG-$_D$(KLAKLAK)$_2$ and (RGD-4C)-GG-$_D$(KLAKLAK)$_2$

Given our results in culture, we proceeded to test both targeted pro-apoptotic peptides *in vivo*, using nude mice with human MDA-MD-435 breast carcinoma xenografts. Tumor volume in the groups treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ was on average 10% that of control groups (Fig. 6a); survival was also longer in these groups than in control groups (Fig. 6b). The control was a non-targeted 'mimic' CARAC-GG-$_D$(KLAKLAK)$_2$ peptide; the CARAC sequence has a charge, size and general structure similar to that of CNGRC. Some of the mice treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ outlived control mice by several months, indicating that both primary tumor growth and metastasis were inhibited by CNGRC-GG-$_D$(KLAKLAK)$_2$. Treatment in nude mice bearing MDA-MD-435 breast carcinoma xenografts with (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ also resulted in a significantly reduced tumor and metastatic burden (Fig. 7). Experimental parameters included tumor volumes before and after treatment (Fig. 7a), wet weights of the tumors (Fig. 7b, right) and weight of lung metastases (Fig. 7b, left). The control group was treated with an equimolar mixture of RGD-4C and $_D$(KLAKLAK)$_2$. Histopathological and TUNEL analysis showed cell death in the treated tumors and evidence of apoptosis and necrosis (data not shown).

To assess toxicity in mice without tumors, we have administered CNGRC-GG-$_D$(KLAKLAK)$_2$ or (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ to both immunocompetent (balb/c) and to immunodeficient (balb/c nude) mice at a dose of 250 µg/mouse per week for eight doses. No apparent toxicities have been found in 3 months. Moreover, in these conditions, the peptides are not immunogenic, as determined by ELISA of blood obtained from the immunocompetent mice (data not shown).

We have also evaluated the stability of the CNGRC-GG-$_D$(KLAKLAK)$_2$ and (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ peptides *ex vivo* and in mice. We analyzed the two targeted peptides using mass spectrometry. In the first set of experiments, the targeted peptides were pre-mixed with whole blood and incubated at 37 °C.
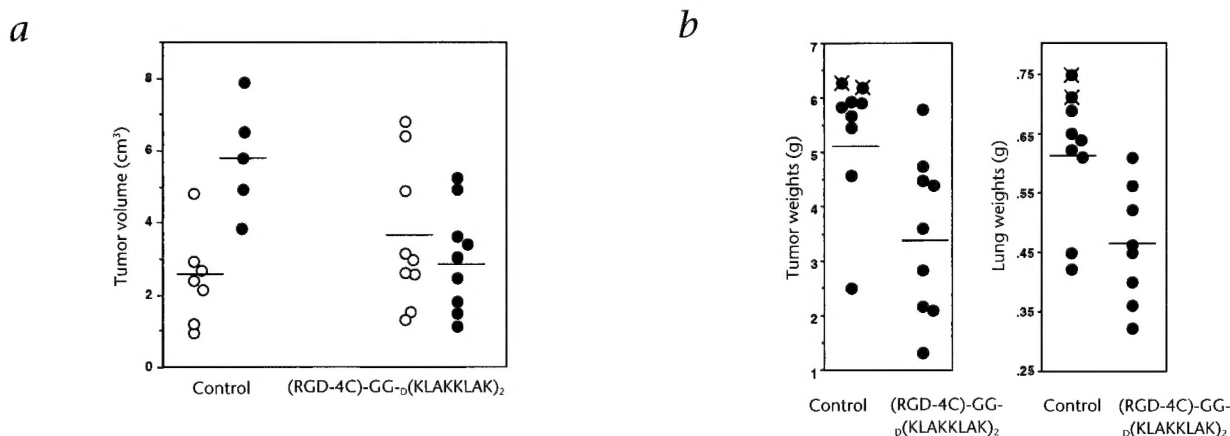
with CNGRC-GG-$_D$(KLAKLAK)$_2$ showed abnormal condensation and vacuolization despite a relatively preserved cell morphology (Fig. 5d–f, black arrows). Progressive cellular damage could be seen after 24 hours, when many mitochondria showed ultrastructural features of low-level oxidative phosphorylation (Fig. 5g and h); in later stages, some of the damaged mitochondria (Fig. 5g, black arrows) showed profound changes, as seen in the isolated mitochondria treated with $_D$(KLAKLAK)$_2$ (Fig. 2c, right lower panel). In some cells, this process progressed to a late apoptotic stage. Typical vacuolization and condensed nuclei became evident (Fig. 5i). These results show that the mitochondria underwent changes in morphology and function that were well-represented by a progression from a state of normal morphology and normal oxidative phosphorylation (Fig. 5a) to a state of condensed morphology and a high rate of oxidative phosphorylation (Fig. 5d) to a final edemic state (Fig. 5g) associated with a low energy level.



**Fig. 7** Treatment of nude mice bearing MDA-MB-435-derived human breast carcinoma xenografts with (RGD-4C)-GG-$_D$(KLAKLAK)$_2$. **a**, Tumors treated with (RGD-4C)-GG-$_D$(KLAKLAK)$_2$ are smaller than control tumors treated with an equimolar mixture of RGD-4C peptide and $_D$(KLAKLAK)$_2$. Tumor volumes were assessed on day 1 (O) and day 90 (●). $P$ = 0.027, $t$-test. **b**, Tumor weights (right) and lung metastatic burden (left) are also decreased in mice treated with (RGD-4C)-GG-$_D$(KLAKLAK)$_2$; these were measured when the experiment ended, on day 110 ($n$ = 9 animals/group). $P$< 0.05, $t$-test.

The peptides were intact up to 1 hour in these conditions. In the second set of experiments, mice were injected intravenously with the two targeted peptides and blood samples were analyzed; the peptides were present at 10 minutes after administration (data not shown). We chose these short circulation times to coincide with the experimental conditions established for 'homing' of targeted peptides *in vivo*[5,7,8].

Targeted pro-apoptotic peptides represent a potential new class of anti-cancer agents; their activity may be optimized for maximum therapeutic effect by adjusting properties such as residue placement, domain length, peptide hydrophobicity and hydrophobic moment[29]. Beyond this, future targeted pro-apoptotic peptides might be designed to disrupt membranes using a completely different type of pro-apoptotic domain such as β-strand/sheet-forming peptides[30]. Our results provide a glimpse at a new cancer therapy combining two levels of specificity: 'homing' to targeted cells and selective apoptosis of such cells after entry.

## Methods

**Reagents.** Human recombinant vascular endothelial growth factor (VEGF; PharMingen, San Diego, California), antibody against caspase-3 (Santa Cruz Biotechnology, Santa Cruz, California), streptavidin FITC (Sigma) and N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; BioMol, Plymouth Meeting, Pennsylvania) were obtained commercially. Peptides were synthesized to our specifications at greater than 90% purity by HPLC (DLSLARLATARLAI, Coast (San Diego, California); all other peptides, AnaSpec (San Jose, California).

The computer-generated model was made with Insight II (Molecular Simulations, San Diego, California) running on an $O_2$ work station (Silicon Graphics, Mountain View, California)

**Cell culture.** Dermal microvessel endothelial cells (DMECs) were grown in CADMEC Growth Media™ (media and cells from Cell Applications, San Diego, California). DMECs were then cultured in three experimental conditions: proliferation (30% confluency in a growth media supplemented with 500 ng/ml VEGF); no proliferation (100% confluency in media formulated to maintain a monolayer); and cord formation (60% confluency (required for induction) in media formulated to induce cord formation). KS1767 and MDA-MB-435 cells were cultured as described[5,8,27,28].

**Internalization assay.** KS1767 cells grown on coverslips were treated with 100 µM biotin-labeled CNGRC or biotin-labeled CARAC (negative control) for 24 h. Streptavidin FITC was added to the coverslips, and cells were then viewed on an inverted microscope (Nikon TE 300) using a FITC filter.

**Mitochondrial swelling assays.** Rat liver mitochondria were prepared as described[10]. The concentrations used were 10 µM $_D$(KLAKLAK)$_2$, 10 µM DL-SLARLATARLAI (negative control), or 200 µM $Ca^{2+}$ (positive control). The peptides were added to mitochondria in a cuvette, and swelling was quantified by measuring the optical absorbance at 540 nm.

**Cell-free apoptosis assays.** Cell-free systems were reconstituted as described[10]. For the mitochondria-dependent reactions, rat liver mitochondria were suspended in normal (non-apoptotic) cytosolic extracts of DMECs. The peptides were added at a concentration of 100 µM. After incubation for 2 h at 30 °C or 37 °C, mitochondria were removed by centrifugation, and the supernatant was analyzed by SDS–PAGE and immunoblotting (12% gels, BioRad, Richmond, California). Proteins were transferred to PVDF membranes (BioRad, Richmond, California) and incubated with antibody against caspase-3, followed by ECL detection (Amersham).

**Caspase activity of cell lysates.** The caspase activity of DMEC lysates was measured as described[10]. Aliquots of cell lysates (1 µl lysate; 8–15 mg/ml) were added to 100 µM DEVD-pNA (100 µl; 100 mM HEPES, 10% sucrose, 0.1% CHAPS and 1 mM DTT, pH 7.0). Hydrolysis of DEVD-pNA was monitored by spectrophotometry (400 nm) at 25 °C.

**Morphological quantification of cellular apoptosis.** Percent viability and $LC_{50}$ (Table 1) were determined by apoptotic morphology[10]. For the percent viability assay, DMECs were incubated with 60 µM active peptide or control peptide. Cell culture medium was aspirated at various times from adherent cells, and the cells were gently washed once with PBS at 37 °C. Then, a 20-fold dilution of the dye mixture (100 µg/ml acridine orange and 100 µg/ml ethidium bromide) in PBS was gently pipetted on the cells, which were viewed on an inverted microscope (Nikon TE 300). The cell death seen was apoptotic cell death and was confirmed by a caspase activation assay. Not all cells progressed through the stages of apoptosis at the same time. At the initial stages, a fraction of the cells were undergoing early apoptosis. At later stages, this initial fraction had progressed to late apoptosis and even to the necrotic-like stage associated with very late apoptosis (for example, loss of membrane integrity in apoptotic bodies). However, these cells were joined by a new fraction undergoing early apoptosis. Thus, cells with nuclei showing margination and condensation of the chromatin and/or nuclear fragmentation (early/mid-apoptosis; acridine orange-positive) or with compromised plasma membranes (late apoptosis; ethidium bromide-positive) were considered not viable. At least 500 cells per time point were assessed in each experiment. Percent viability was calculated relative to untreated controls. $LC_{50}$ for monolayer, proliferation (60% confluency), and cord formation were assessed at 72 h.

**Mitochondrial morphology.** DMECs after 24 and 72 h of treatment with peptide were incubated for 30 min at 37 °C with a mitochondrial stain (100 nM MitoTracker Red™ CM-H$_2$XRos; the nonfluorescent, reduced form of the compound) and a nuclear stain (500 nm DAPI; Molecular Probes, Eugene, Oregon). Mitochondria were then visualized under fluorescence microscopy (100x objective) under an inverted microscope using a triple wavelength filter set (Nikon).

**Electron microscopy.** Rat liver mitochondria were prepared as described[10]. The mitochondria were incubated either with a control peptide (DLSLAR-LATARLAI) or with 3 µM $_D$(KLAKLAK)$_2$. The effects of the treatment were assessed at different times (Fig. 2c). Kaposi sarcoma cells were collected from 24-well Biocoat Cell culture inserts for electron microscopy (Becton Dickinson, Franklin Lakes, New Jersey). Cell monolayers at 80% confluency were exposed to either 100 µM CARAC-GG-$_D$(KLAKLAK)$_2$ (control) or CNGRC-GG-$_D$(KLAKLAK)$_2$ (targeted) (Fig. 5). All specimens were fixed with 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4 for 30 to 45 min at the room temperature, followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration using a graded series of ethanol rinses, tissues were embedded in resin. Ultrathin sections after additional counterstainings were viewed and photographed on an electron microscope (Hitachi H-600).

**Human tumor xenografts.** MDA-MB-435-derived tumor xenografts were established in female nude mice 2 months old (Jackson Labs, Bar Harbor, Maine) as described[33]. The mice were anesthetized with Avertin as described[31]. The peptides were administered at a dose of 250 µg/week per mouse, given slowly through the tail vein in a volume of 200 µl. Three-dimensional measurements of tumors were made by caliper on anesthetized mice, and were used to calculate tumor volume[5,8]. Then, tumors and lungs were surgically removed and the wet weights recorded. Animal experimentation was reviewed and approved by the Institute's Animal Research Committee.

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